Progesterone Enhances Macrophage Colony-Stimulating Factor(M-CSF) Production in Human Endometrial Stromal Cells in vitro(Dissertation_全文)

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(子宮内膜間質細胞に於けるＭ－ＣＳＦ産生とその卵巣性ステロイドホルモンによる調節)

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Progesterone Enhances Macrophage Colony-Stimulating Factor Production in Human Endometrial Stromal Cells *in Vitro**

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ABSTRACT

Increasing evidence suggests that macrophage colony-stimulating factor (M-CSF) is produced in the uterine endometrium and that it plays an important role in the reproductive process. In the present study, using an *in vitro* decidualization model and human endometrium, we investigated M-CSF messenger RNA (mRNA) expression in human endometrial stromal cells (ESC) by Northern blotting and *in situ* hybridization. The secreted M-CSF in the culture medium of ESC was measured by enzyme-linked immunosorbent assay. ESC were cultured in the presence of progesterone (P) or estrogen. After a 9-day culture with P, when *in vitro* decidualization was confirmed by the production of PRL, M-CSF mRNA and protein levels were 3.1 ± 0.5- and 3.2 ± 0.8-fold (mean ± SEM) higher, respectively, than those in cultures without P ($P < 0.01$). The P-induced increase was dose dependent. On the other hand, estrogen did not increase M-CSF mRNA expression. M-CSF mRNA expression in the first trimester deciduae that expressed PRL mRNA was higher than that in the endometria. By *in situ* hybridization, ESC as well as epithelial cells were shown to express M-CSF both *in vitro* and *in vivo*. These findings indicate that human ESC (decidua cells) express M-CSF mRNA and suggest that they secrete M-CSF in a P-dependent manner during the process of decidualization. (*Endocrinology* **135:** 1921–1927, 1994)

THE ENDOMETRIUM has a biological role in achieving successful implantation through secretory changes in the glandular epithelium and decidual changes in the stromal cells. These changes are primarily controlled by ovarian steroid hormones, but in addition, the endometrial stromal cells (ESC) may be regulated by some cytokines (1–3). ESC also reportedly secrete some cytokines in response to ovarian steroids (4, 5). Macrophage colony-stimulating factor (M-CSF; also referred to as CSF-1) is a growth factor that is produced by macrophages, fibroblasts, and endothelial cells. This cytokine was originally identified as a regulating factor of mononuclear phagocytic cells (6, 7). M-CSF has been identified in human (8–12) and mouse (13–18) endometrial tissues and placenta. The action of M-CSF is mediated by a specific membrane receptor encoded by the c-*fms* protooncogene (19). This M-CSF receptor, c-*fms*, is also present in human (10, 20, 21) and mouse (14, 15, 18) endometrium and placenta. These reports suggest that M-CSF plays some role in endometrial and placental function. However, in the human uterus, M-CSF is reportedly localized in uterine epithelial cells (11, 12), not in stromal cells. In this study, by Northern blotting and *in situ* hybridization for gene expression and enzyme-linked immunosorbent assay (ELISA) for production, we showed that human ESC (decidual cells) also expressed M-CSF messenger RNAs (mRNAs) and produced M-CSF in a progesterone (P)-dependent manner during the process of decidualization.

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Materials and Methods

Specimens

Human endometria were obtained from 19 patients, aged 38–48 yr, who had undergone hysterectomy for the treatment of uterine myoma. A small portion of the endometrial tissue from each specimen was examined histologically and dated according to the criteria of Noyes *et al.* (22). Fourteen specimens were late proliferative, and 5 were secretory. First trimester human deciduae (n = 5) were obtained from patients who had undergone legal abortions. Human placental tissue (n = 1) was obtained from a patient who had undergone elective cesarean section. Informed consent was obtained from all patients.

Cell cultures

The ESC from the proliferative endometrial tissues were isolated as previously described (2). Briefly, specimens were washed, finely minced, and enzymatically digested. After subsequent pipetting, the cell suspension, diluted twice with RPMI-1640, was placed in the centrifugation tube (Corning Glass Works, Corning, NY) and left upright for 10 min at unit gravity. The supernatant, excluding the lowermost 2 ml, was transferred into a new tube to collect suspended single cells. After repeating this procedure several times, the cell suspension was washed three times and used as the ESC. The viability, determined by dye exclusion, was at least 90%. Two million viable stromal cells were cultured in 25-ml flasks in RPMI-1640 (Gibco, Grand Island, NY) with 10% fetal calf serum (Dainippon Pharmaceutical Co., Osaka, Japan), 100 IU/ml penicillin, and 100 μg/ml streptomycin for 3–12 days at 37 C in a humidified atmosphere of 5% CO_2 in air. To determine the effects of ovarian steroids, P (10^{-6} M) or 17β-estradiol (E; 10^{-8} M) dissolved in ethanol or vehicle alone was added to the medium from the beginning

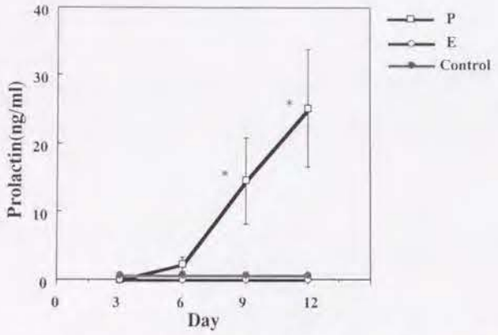

FIG. 1. The time course of PRL concentration in the medium of cultured human ESC. Human ESC were cultured with P (10^{-6} M), E (10^{-8} M), or vehicle alone (Control) for the indicated number of days. The PRL concentration was measured by RIA. Each value represents the mean ± SEM of nine separate cultures with P and four with E. *, $P < 0.05$.

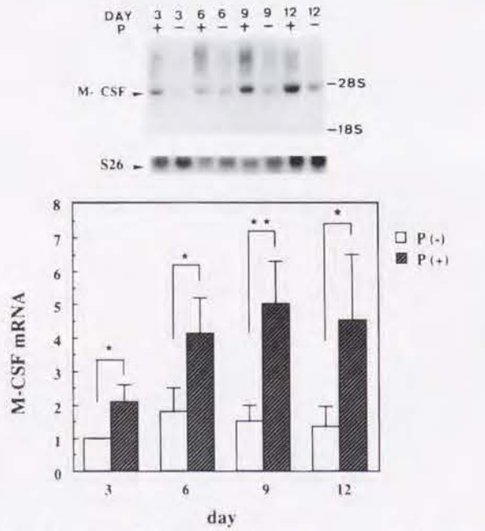

FIG. 2. The effect of P on M-CSF mRNA expression in cultured human ESC. Human ESC were cultured with or without P (10^{-6} M) for the indicated number of days. Twenty micrograms of total RNA extracted from these tissues were hybridized with the probes for M-CSF and S26 ribosomal protein (*top*). The densitometric analysis of M-CSF mRNA was normalized by the S26 protein and expressed relative to P (−) on day 3. Each value represents the mean ± SEM of four separate cultures (*bottom*). During the time-course study, the M-CSF mRNA level was significantly higher with than without P treatment ($P < 0.05$, by analysis of variance; *, $P < 0.05$; **, $P < 0.01$, by Fisher's protected least significant difference test).

of the culture. The concentration of ethanol did not exceed 0.1% of the total volume. Culture media were changed every 3 days and stored at −20 C until the ELISA and PRL assay. At the completion of culture, the cells were collected for the isolation of total RNA. To analyze the time-dependent effects of ovarian steroids, ESC were cultured with E (10^{-8} M) or P (10^{-6} M), and total RNA was extracted every 3 days until day

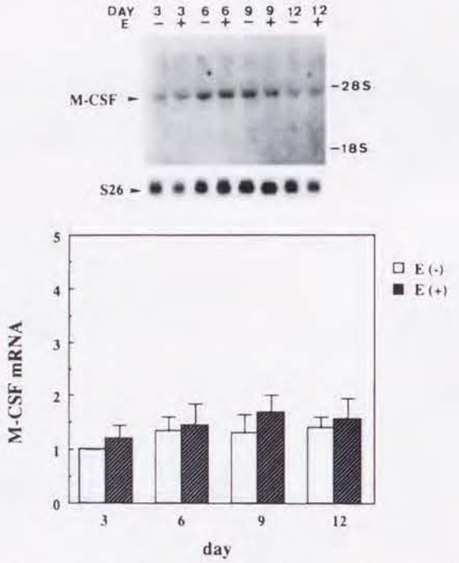

FIG. 3. The effect of E on M-CSF mRNA expression in cultured human ESC. Human ESC were cultured with or without E (10^{-8} M) for the indicated number of days. Twenty micrograms of total RNA extracted from these tissues were hybridized with the probes for M-CSF and S26 ribosomal protein (*top*). The densitometric analysis of M-CSF mRNA was normalized by the S26 protein and expressed relative to E (−) on day 3. Each value represents the mean ± SEM of four separate cultures (*bottom*). The M-CSF mRNA level with E was not different from that without E.

12. The dose-dependent effects of P or E were examined after 9 days of culture with various doses of P (10^{-8}-10^{-6} M) or E (10^{-10}-10^{-8} M).

Flow cytometric analysis

Flow cytometry was performed as described previously (23). The antibodies were LeuM3 (Becton Dickinson, Mountain View, CA) for macrophages, anticytokeratin for epithelial cells, anti-von Willebrand factor for endothelial cells, and CD10 antibodies (Nichirei Co., Tokyo, Japan) for stromal cells. CD10 antigens are reportedly expressed on human ESC (23). The negative controls were cells stained with fluorescein isothiocyanate-conjugated second antibody alone. At least 10,000 cells were analyzed for each specimen.

PRL assay by RIA and M-CSF assay by ELISA

The PRL concentration in the culture medium was measured by RIA using a commercial kit (Daiichi Radioisotope Lab., Tokyo, Japan). The detection limit was 1.0 ng/ml, and the intra- and interassay coefficients of variation were between 1.9–7.1% and 1.6–3.6%, respectively. The concentrations of secreted M-CSF in the culture medium were measured by the ELISA established by Hanamura *et al.* (24). The detection limit was 10 U/ml, and the coefficient of variation was 7.5%. Each assay was performed in duplicate. These data were analyzed by paired *t* test.

In situ hybridization

In situ hybridization was performed as described previously by Kaneko *et al.* (25). Briefly, a plasmid containing the human M-CSF complementary DNA (cDNA) was linearized with the appropriate enzymes to generate sense and antisense templates. Digoxigenin-labeled

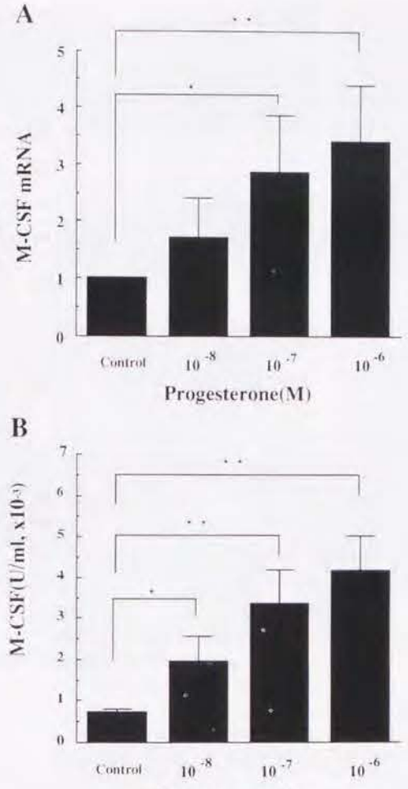

FIG. 4. The dose-dependent effect of P on M-CSF mRNA expression and M-CSF production in cultured human ESC. Human ESC were cultured with vehicle alone or with various doses of P (10^{-6}-10^{-8} M) for 9 days. A, The densitometric analysis of M-CSF mRNA expression was normalized by ribosomal protein S26 mRNA expression. Each value represents the mean ± SEM of four culture samples. P increased M-CSF mRNA levels in a dose-dependent manner. B, The concentrations of secreted M-CSF in the culture medium were measured by ELISA. Each value represents the mean ± SEM of three culture samples. P increased M-CSF production in a dose-dependent manner ($P < 0.05$, by analysis of variance; *, $P < 0.05$; **, $P < 0.01$ *vs.* control, by Scheffe's F test).

single strand RNA probes were transcribed with T3 and T7 RNA polymerases using a DIG RNA Labeling Kit (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany). Human nonpregnant endometrial tissues were fixed with freshly prepared 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 24 h and embedded in paraffin wax after dehydration by the conventional method. Human ESC were cultured for 9 days in Lab-Tek chamber slides (Nunc, Naperville, IL) with P (10^{-6} M). Before hybridization, the culture supernatant was removed, and the cells were washed with PBS, then fixed with 4% paraformaldehyde diluted in PBS for 15 min. They were successively exposed to proteinase-K, 0.2 N HCl, and 0.25% acetic anhydride in 0.1 M triethanolamine. Thereafter, they were dehydrated with a series of graded ethanol and air dried. Hybridization was carried out overnight at 50 C with about 0.5 mg/ml digoxigenin-labeled RNA probes in 50% formamide, 10% dextran sulfate, 10 × Denhardt's solution (5 g Ficoll, 5 g polyvinylpyrrolidone, 5 g BSA, and H_2O to 500 ml), 600 mM NaCl, and 250 μg/ml *Escherichia coli* transfer RNA. After hybridization, the slides were digested with ribonuclease-A (3 μg/ml) at 37 C for 30 min, then washed twice with 2 × SSC (1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate) and 0.2 × SSC for 20 min each at 50 C. The hybridized digoxigenin-labeled probes were detected using a Nucleic Acid Detection Kit (Boehringer Mannheim, Indianapolis, IN). Hybridization with the sense probe at the same time under identical conditions served as the negative control.

Northern blotting

Total RNA from human endometria (n = 10; 5 proliferative and 5 secretory), first trimester human deciduae (n = 5), and cultured ESC was isolated by centrifugation through 4 M guanidinium isothiocyanate and 5.7 M CsCl (26). The probe for M-CSF was an 810-basepair *Sma*I-*Eco*RI fragment (nucleotides 1010–1820) of human M-CSF cDNA (27). The probe for decidual PRL was prepared as follows. Two micrograms of placenta total RNA were reverse transcribed with a random primer (Pharmacia-LKB, Tokyo, Japan). The resulting cDNA mixture was amplified by 40 cycles of PCR with human PRL-specific primers (sense primer, 5′-TGCTGCTGCTGGTGTCAA-3′; antisense primer, 5′-GAA-ATGGATG TGGGCTTAG-3′). The PCR product was cloned into the pBluescript SK(−) plasmid (28), and the insert was verified by sequencing (29). The insert was purified and used as the human decidual PRL probe in Northern blotting.

Twenty micrograms of total RNA were electrophoresed on a 1.5% agarose gel and transferred to nylon membranes, which were then incubated with prehybridization solution (Quick Hyb, Stratagene, La Jolla, CA) for 15 min at 68 C and hybridized with the labeled probes. The probes for M-CSF and PRL were labeled by random primer labeling (30) to a specific radioactivity of 0.8–1.0 × 10^9 cpm/μg DNA. Hybridization with the labeled probe was performed for 1 h at 68 C. After hybridization, the membranes were washed at room temperature in 0.2 × SSC plus 0.1% sodium dodecyl sulfate, followed by 0.1 × SSC and 0.1% sodium dodecyl sulfate at 65 C for 20 min, and autoradiographed. The membranes were washed and used again for Northern blotting, with human ribosomal protein S26 as an internal control, because its expression level is virtually constant in many tissues (31). The mRNA level was calculated on the basis of the hybridized signal measured by densitometric scanning of the autoradiograph.

Statistics

Statistical differences between sample means were calculated by analysis of variance, followed by Fisher's protected least significant differences test. The results are expressed as the mean ± SEM, and $P < 0.05$ was considered significant.

Results

Purity of ESC

We measured the purity of the ESC fraction by flow cytometry. The fraction contained about 2–3% epithelial cells, 1–2% macrophages, and 95% stromal cells, and the contamination by endothelial cells was negligible after 3 days of culture. The ratio of epithelial cells decreased to less than 2% after 9 days of culture. The ratio of contaminating macrophages in the control group without steroids and the E and P groups was 0.9–1.6%, 0.7–0.8%, and 0.7–0.9%, respectively, on day 9 of culture. The ratio of macrophages was very low and not affected by the culture with either E or P.

PRL production by cultured ESC

In the presence of P, PRL was first immunologically detectable in culture medium after a lag of 5–6 days. Subsequently, the level of PRL increased sharply to reach 10–40

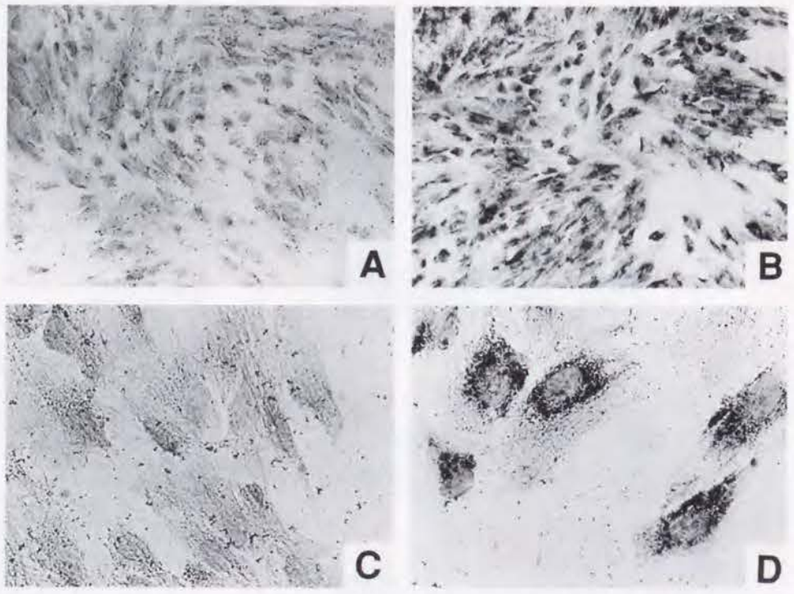

FIG. 5. *In situ* hybridization of cultured ESC with the antisense RNA probe for M-CSF mRNA (B and D) and the control sense probe (A and C). The antisense RNA probe revealed that the transcript for M-CSF mRNA was abundant in the stromal cells cultured in the presence of P for 9 days. Original magnification: A and B, ×120; C and D, ×1540.

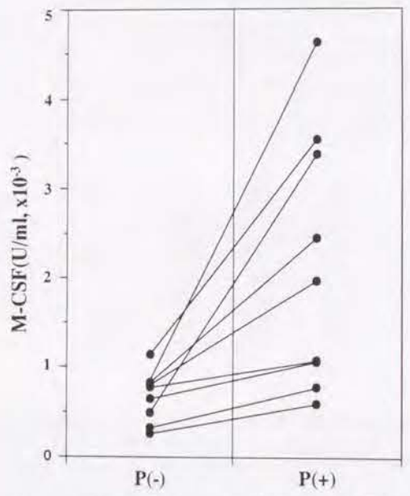

FIG. 6. The concentrations of secreted M-CSF in the culture medium. Human ESC were cultured with or without P (10^{-6} M) for 9 days. Nine paired culture media with or without P were collected, and the M-CSF concentration was measured by ELISA. P increased M-CSF secretion 3.20 ± 0.6-fold compared with that in its absence ($P < 0.01$, by paired *t* test).

ng/ml at the completion of culture (Fig. 1). On the other hand, PRL was undetectable in the presence of E. Morphologically, decidualization was characterized by the conversion of spindle-shaped stromal cells into epithelial-like cells with enlarged nuclei and an increased amount of cytoplasm (2, 32). *In vitro* decidualization of cultured ESC was confirmed by PRL production and morphological transformation after 6–9 days of culture with P. When the decidualization was confirmed, the number of ESC increased 1.45-fold after P and 1.4-fold after E.

Effects of P or E on M-CSF mRNA levels in cultured ESC

To examine the effects of ovarian steroids on the M-CSF mRNA level, human ESC were cultured with P (10^{-6} M) or E (10^{-8} M) for 3–12 days. M-CSF mRNA in ESC was evident as a single hybridization band of about 4.0 kilobases (kb). In the presence of P, the M-CSF mRNA level increased from day 3 and appeared to reach a plateau after 9 days (Fig. 2). The M-CSF mRNA level in the control group was not affected by the length of culture. After 12 days in culture, the M-CSF mRNA level was significantly higher in the presence of P than in its absence ($P < 0.05$–0.01). In contrast, E did not affect the M-CSF mRNA level (Fig. 3). To analyze the dose dependence of the increase in M-CSF mRNA and production levels, ESC were incubated with various doses of P or E for 9 days. P increased the M-CSF mRNA levels in a dose-dependent manner, with 10^{-8}, 10^{-7}, and 10^{-6} M P resulting in 1.7 ± 0.7-, 2.9 ± 1.1-, and 3.4 ± 1.1-fold increases, respectively, compared to the level without P (Fig. 4A). P also increased the production level in a dose-dependent manner (Fig. 4B). E did not affect M-CSF mRNA expression at any examined doses (data not shown).

Detection of M-CSF producing cells in vitro

In situ hybridization of P-treated ESC with the antisense RNA probe revealed that the transcript for M-CSF mRNA was abundant in more than 90% of the cultured cells (Fig. 5, B and D). The control sense probe did not significantly stain the cells (Fig. 5, A and C). As the purity of ESC was over 95%, the cells expressing M-CSF mRNA may not be contaminants, but stromal cells themselves.

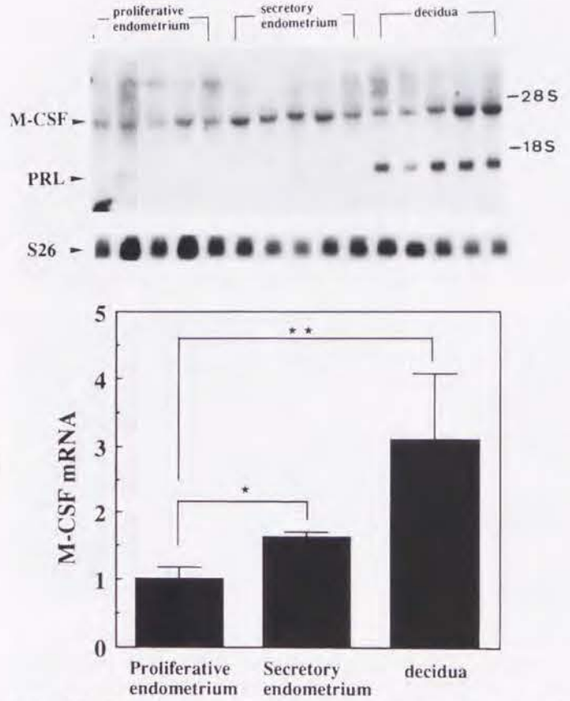

FIG. 7. M-CSF and PRL mRNA expression in human endometrial and decidual tissues. Total RNA was extracted from 10 human endometrial tissues (5 proliferative and 5 secretory) and 5 first trimester human deciduae, then hybridized with probes for M-CSF, PRL, and S26 ribosomal protein (*top*). The densitometric analysis of M-CSF expression was normalized by S26 protein expression (*bottom*). M-CSF mRNA levels in secretory phase endometrium and first trimester decidua were higher than that in proliferative phase endometrium ($P < 0.05$, by analysis of variance; *, $P < 0.05$; **, $P < 0.01$, by Fisher's PLSD). There was no significant difference in M-CSF mRNA levels between secretory phase endometrium and decidua. Decidual PRL mRNA was expressed only in first trimester deciduae as a single hybridization band of about 1.1 kb.

M-CSF secretion by cultured ESC

After 9 days in culture, when *in vitro* decidualization was confirmed, nine pairs of test specimens treated with or without P were measured for M-CSF. The concentrations of M-CSF without P were in the range of 252–1134 U/ml (mean ± SE, 673 ± 92). M-CSF with P were in the range of 594–4622 IU/ml (mean ± SE, 2159 ± 476; Fig. 6). P increased M-CSF secretion in cultured ESC 3.20 ± 0.6-fold compared with that in the absence of P ($P < 0.01$).

M-CSF and PRL expression in the human endometrium and decidua

All of the examined samples from the human endometria and human deciduae expressed M-CSF mRNA (Fig. 7). M-CSF mRNA levels in secretory phase endometrium and first trimester decidua were higher than that in the proliferative phase endometrium ($P < 0.05$ and $P < 0.01$, respectively). There was no significant difference in the M-CSF mRNA levels between the secretory phase endometrium and the decidua. Decidual PRL mRNA expression was detected only in the first trimester deciduae as a single hybridization band of about 1.1 kb (Fig. 7).

Detection of M-CSF-producing cells in the nonpregnant human endometrium

To identify the cells expressing M-CSF mRNA in the human endometrium, *in situ* hybridization was carried out. The antisense RNA probe revealed that the stromal cells expressed M-CSF mRNA as well as the epithelial cells (Fig. 8, B and D). The control sense probe did not significantly stain the cells (Fig. 8, A and C).

Discussion

Using the *in vitro* decidualization model of human ESC, we examined M-CSF mRNA expression and its hormonal regulation. The separated ESC, when cultured with P, underwent the morphological changes and produced PRL, indicating that the *in vitro* transformation of ESC by P mimics *in vivo* decidualization. Using this *in vitro* system, we demonstrated that P increased M-CSF gene expression and production in a dose-dependent manner. M-CSF secretion in the culture medium increased as well as mRNA expression. The effect of P was evident within the range of its physiological concentrations that circulate during the luteal phase and in early pregnancy, that is at the time of endometrial decidualization. The finding that M-CSF mRNA expression in secretory phase endometrium and first trimester decidua, which is affected by ovarian P, was higher than that in the proliferative phase endometrium was consistent with the *in vitro* results. On the contrary, E did not affect the expression of M-CSF mRNA. This is the first evidence to show that human ESC (decidual cells) produce M-CSF in a P-dependent manner.

The importance of M-CSF in the reproductive process has been suggested by studies of the osteopetrotic (*op/op*) mutant mouse, which lacks M-CSF entirely and shows infertility in a homozygous (*op/op Xop/op*) mutant cross and reduced implantation numbers in a heterozygous cross (16). The exact reason for the infertility in the *op/op* mouse is not clear, but the disturbance of implantation due to the lack of M-CSF in the uterus may be one of the causes. Oocytes express c-*fms*, the M-CSF receptor mRNA, during their maturation (18), and supplementation of the embryo culture medium (M6 medium) with M-CSF significantly enhances the development of two-cell embryos to blastocysts (17). By *in situ* hybridization, we demonstrated that the stromal cells in

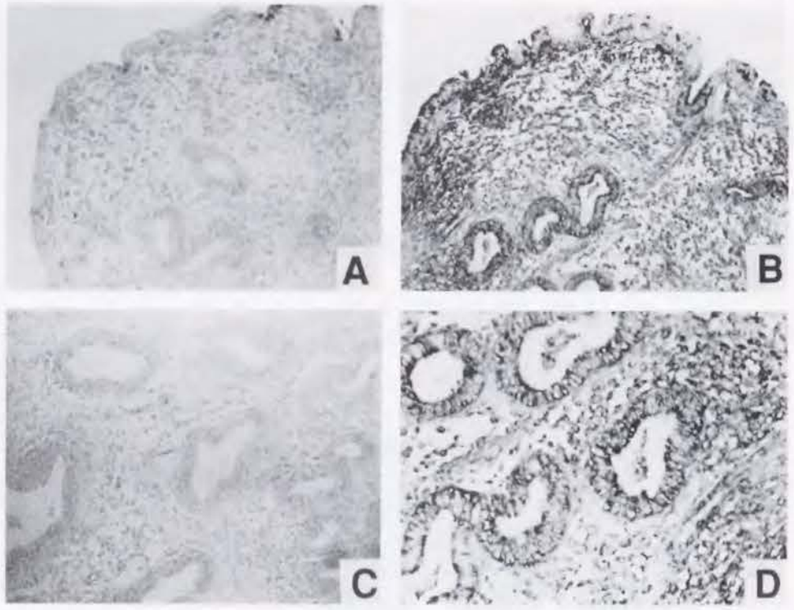

FIG. 8. *In situ* hybridization of nonpregnant endometrium with the antisense RNA probe for M-CSF mRNA (B and D) and the control sense probe (A and C). The antisense RNA probe revealed that the transcript for M-CSF mRNA was abundant in epithelial cells and moderate in stromal cells.

placental villi express M-CSF mRNA and suggested a role for M-CSF as a growth factor for the tissue macrophages and trophoblasts that express c-*fms* (10). In the present study, we showed that the ESC produce M-CSF. This suggests that the M-CSF from the ESC also works as a growth factor in the feto-maternal interface, because the implanted embryo expressing c-*fms* is surrounded by the stromal cells (decidual cells). Moreover, the P-dependent increase in M-CSF mRNA in the stromal cells suggests the importance of M-CSF during the luteal phase and early in pregnancy, when the P from the corpus luteum affects the uterus. As for the response to P in M-CSF production, considerable variabilities have been observed among the samples. The same variabilities have been detected in the deciduae *in vivo*. There may exist some other factors in addition to P that affect M-CSF production in human ESC and decidual cells.

In this study, we also examined the relationship between decidualization and M-CSF gene expression. In the *in vitro* system, PRL production and morphological changes in the ESC occurred on day 9, whereas the level of M-CSF mRNA increased on day 3. *In vivo*, the level of M-CSF mRNA expression in the secretory phase endometrium that did not yet express the PRL mRNA was also higher than that in the proliferative endometrium. These findings indicate that the increase in M-CSF production precedes the decidualization changes both *in vivo* and *in vitro*. Whether M-CSF induction by P is a prerequisite for PRL production in the ESC remains to be determined.

In the human endometrium, epithelial cells have been reported to produce a large amounts of M-CSF by immunohistochemistry (12) or PCR (11). However, in these reports, M-CSF was detected only in the epithelial cells, not in the stromal cells. In this study, the cultured ESC was shown to express M-CSF mRNA. The contaminating epithelial cells in the *in vitro* culture may have produced the M-CSF. However, this is unlikely, because the ratio of epithelial cells was very low, and it decreased, whereas gene expression and secretion of M-CSF increased in the presence of P. Another possibility is that contaminating macrophages expressed M-CSF mRNA. However, judging from the result of *in situ* hybridization, in which most of the cultured cells expressed M-CSF, the stromal cells (decidual cells) produced M-CSF under our conditions *in vitro*. In fact, by *in situ* hybridization of the endometrium, the ESC was shown to express M-CSF mRNA as well as epithelial cells. The expression of M-CSF in ESC may have been lower than the sensitivity of the immunohistochemical methods previously employed.

In conclusion, although M-CSF is thought to play important roles in various reproductive processes as a local mediator, this study revealed that the human ESC (decidual cells) are also an important source of this cytokine and suggested that the expression of M-CSF in ESC was under the control of P in the process of decidualization. The roles that ESC (decidual cells) play in M-CSF production and early pregnancy *in vivo* and the biological actions of M-CSF within the female reproductive tract should be studied further.

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